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Spotlight on chimeric antigen receptor engineered T cell research and clinical trials in China

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T cell mediated adoptive immune response has been characterized as the key to anti-tumor immunity. Scientists around the world including in China, have been trying to harness the power of T cells against tumors for decades. Recently, the biosynthetic chimeric antigen receptor engineered T cell (CAR-T) strategy was developed and exhibited encouraging clinical efficacy, especially in hematological malignancies. Chimeric antigen receptor research reports began in 2009 in China according to our PubMed search results. Clinical trials have been ongoing in China since 2013 according to the trial registrations on clinicaltrials.gov.. After years of assiduous efforts, research and clinical scientists in China have made their own achievements in the CAR-T therapy field. In this review, we aim to highlight CAR-T research and clinical trials in China, to provide an informative reference for colleagues in the field.

chimeric antigen receptor, tumor associated antigen, adoptive cell therapy, gene modification, combination strategy

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INTRODUCTION

In recent years, chimeric antigen receptor engineered T cell (CAR-T) therapy emerged as a leading role in the cancer immunotherapy arena (Yang, 2015). The excitement surrounding CAR-T therapy is kindled by thrilling objective results from clinical trials, owing to years of diligent collaborative study from laboratory and clinical scientists. CAR-T was originally proposed by Zelig Eshhar and colleagues working at the Weissman Institute in Israel 27 years ago (Gross et al., 1989). CAR-T combines the recognition advantage of the scFv (single chain fragment variable) from monoclonal antibodies and the tumor lysis ability of individual T cells, which enables engineered T cells to target and kill tumor cells. This MHC (major histocompatibility complex)-independent strategy makes MHC down-regulated tumor cells no longer "invisible" to T cells, and enables the direct triggering of effector functions by CAR molecules (Chmielewski et al., 2013; Oren et al., 2014). Research and clinical trials focusing on CAR-T therapy are in progress worldwide, including in China. According to registrations on clinicaltrials.gov., there are 94 CAR-T cell therapy studies worldwide. China has the second most on-going clinical trials (26 studies) at present. Academic institutions, hospitals, and enterprises in China have launched multiple CAR-T associated studies, with promising outcomes being reported in the past few years. Although in its infancy, CAR-T therapy in China is thriving on challenges and opportunities. In this review, we have summarized the past CAR-T research and clinical trials, and looked into the future of CAR-T therapy in China.

CAR CONSTRUCTS

CAR constructs are mainly composed of four elements,

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including antigen-recognizing scFv, the spacer and *trans*-membrane domain, the co-stimulatory domain and the signaling domain (Sadelain et al., 2013).

The most used scFvs in China are derived from well described antibodies, such as anti-CD19 scFv from FMC63 (Dai et al., 2015) and anti-CD20 scFv from HB-9645 (Wang et al., 2014) etc. (Table 1). The use of these scFv fragments to target the well recognized tumor associate antigens partially assure the specificity and safety, which is preferable for clinical trials. Due to the limited number of antigens for tumor cell targeting, some original scFv fragments targeting potential tumor associate antigens have also been developed in China, such as anti-LMP1 (Epstein-Barr virus (EBV) latent membrane protein 1) scFv for EBV-positive cancer cells (Tang et al., 2014) and anti-CMA1 scFv for chronic myeloid leukemia cells (Wang et al., 2012) (Table 1). Most of the scFv sequences are derived from mouse monoclonal antibodies. To avoid potential immunogenicity, humanized antibodies and human antibodies are being developed (Wang et al., 2012). The strategy of using scFv sequences from the patients' auto-antibodies is proposed to circumvent immunogenicity (Weibo and Zhaoming, 2012). To improve the scFv fragments, scientists abroad screened the affinity of scFvs to control the "on target-off tumor" effect. With respect to the same antigen, low affinity scFvs binding tumor cells rather than healthy tissue are preferred (Cruz, 2014; Zhao et al., 2015). However, given the present technology and the limited human tissue samples, clinical trials are essential for the confirmation of the safety of a given scFv targeting a given antigen.

The usage of the spacer and *trans*-membrane domains of CARs are quite empirical; the former mostly depend on the targeting epitope of the specific scFv, while the structural and biochemical impact of the latter remains largely unknown. The spacers used in China are mainly CH_2CH_3, Fc hinge, and CD8α hinge (Deng et al., 2015; Guo et al., 2015; Wang et al., 2013) (Table 1). Recently, it has been reported that CH_2CH_3 spacer could bind soluble Fcγ-receptors and cause off-target cell lysis (Almåsbak et al., 2015), and that the mutation or deletion of the CH_2 region could obviate this problem. Previous studies showed that the distance of the target epitope from the cell membrane is an important parameter for the design of a spacer. For epitopes proximal to membrane such as CD22, a longer spacer is better; while for other epitopes such as receptor tyrosine kinase-like orphan receptor 1 (ROR1), shorter spacers are preferred (Hudecek et al., 2013; Turtle, 2013). These studies indicate that, to promote the efficiency of CARs, the spacer domain needs to be tailored for each of the different target molecules. The *trans*-membrane domains used in China are mainly derived from CD28 and CD8α (Wang et al., 2014; Zhou et al., 2013). Although comparative results are not yet available, it is presumable that the *trans*-membrane domains in favor of CAR-based immune synapse formation might be more advantageous.

The co-stimulatory domains used in China are mainly CD28, CD137 and inducible costimulate (ICOS) (Gao et al., 2014; Guo et al., 2015; Zheng et al., 2010) (Table 1). CD28 and ICOS are B7 family members, while CD137 is a member of the tumor necrosis factor receptor (TNFR) family. CD28 co-stimulation is essential for naive T cells, and ICOS and CD137 are expressed following the induction of CD28 signaling (Curran et al., 2012). CD28 is superior for IL-2 production, and clonal expansion of CAR-T cells. ICOS can help T cells polarize into TH17/TH1 cells and enhance tumor cell lysis capacity (Finney et al., 2003). CD137 specifically reprogram T cells for multifunctional cytokine secretion and enhanced persistence (Carpenito et al., 2009; Song et al., 2011). CD137 also ameliorates T cell exhaustion induced by the spontaneous signaling of CARs (Long et al., 2015; van der Stegen et al., 2015). Other potential molecules such as OX40 and CD27 are also being used as co-stimulatory domains. OX40 can repress IL-10 secretion and counteract the self-repression of activated T cells (Hombach et al., 2012). CD27 is able to enhance effector function and proliferation, and mediate greater persistence as compared with CD28 (Song et al., 2012). It is generally agreed that second-generation CARs are more effective than those of the first-generation, but there is no agreement on which co-stimulatory molecule is the best or whether third-generation CARs are superior (Turtle, 2014). This is probably due to the use of different scFvs, different tumor models, different expression systems and different CAR expression levels in each laboratory. For each CAR construct, comparisons of the co-stimulatory molecules *in vitro* and *in vivo* are still needed to define the most suitable co-stimulatory domain. The signaling domains used in China are all CD3ζ. CD3ζ is mainly used as the signaling domain worldwide, while FcεRI is less used (Srivastava and Riddell, 2015). Moreover, it is also reported that CD3-ζ conducts superior signaling efficacy of CARs as compared to FcεRIγ (Haynes et al., 2001).

In general, the CAR constructs in China are mainly second-generation CARs that synchronize with their global counterparts. In addition to the three generations of CARs, the fourth-generation CARs or "TRUCKs" have been developed abroad (Chmielewski and Abken, 2015). These new CARs endow T cells with additional cytokine production such as IL-12 (Chmielewski et al., 2011) or IL-15 (Hoyos et al., 2010). Also, other modifications are also introduced into CAR-T cells, such as CD137 co-stimulatory ligand expression (Zhao et al., 2015), bi-specific antigen recognition targeting prostate specific membrane antigen (PSMA) and prostate stem cell antigen (PSCA) (Kloss et al., 2013), and extracelluar matrix degradation with heparanase (Caruana et al., 2015) etc.. Recently, an on-switch CAR paradigm was also developed, which allowed the activation of CAR-T cells to be controlled by small molecules (Wu et al., 2015). Although the next generation strategies help to improve the function of CAR-T cells, they also increase the

Table 1 Published Results on CAR-T therapy in China[a)]

Antigen	Tumor target	Sponsor	Receptor	Culture	Gene transfer	Model	Date
CD123	Acute myeloid leukemia	Anhui Provincial Hospital; Baylor College of Medicine	scFv-IgG4Fc-CD28-CD3zeta	CD3+CD28+IL-2	Retrovi-ruse	*Vitro*	2015
CD138	Multiple myeloma	Chinese PLA General Hospital	scfv-CD8a hinge and TM-CD137-CD3zeta	CD3+IFN-r+IL-2	Lentivirus	Human	2014
CD19	B-cell lineage acute lymphocytic leukemia	Chinese PLA General Hospital	scfv-CD8a hinge and TM-CD137-CD3zeta	CD3+IFN-r+IL-2	Lentivirus	Human	2015
CD20	Chemotherapy refractory advanced diffuse large B cell lymphoma	Chinese PLA General Hospital	scfv-CD8a hinge and TM-CD137-CD3zeta	CD3+IFN-r+IL-2	Lentivirus	Human	2014
CD20	B-cell lymohoma	The First Affiliated Hospital of Wenzhou Medical College	scFv-Fc-CD28-CD3ζ	PHA-L+CD3+IL-2	Electro-poration	*Vitro*	2010
CD33	Acute myeloid leukemia	Chinese PLA General Hospital	scfv-CD8a hinge and TM-CD137-CD3zeta	CD3+IFN-r+IL-2	Lentivirus	Human	2015
EGFR	NSCLC	West China Hospital, Sichuan University	scfv-IgG2 Fc-CD28TM-CD28-CD3zeta	CD3+CD28+IL-2	Electro-poration	NOD/SCID CB-17 mice	2013
EGFR	EGFR positive malignancies	Huaihe Hospital of Henan University	scfv-CD8a hinge and TM-CD28-CD3zeta	CD3+IFN-r+IL-1beta+IL-2	Lentivirus	BALB/c nude	2015
EGFRvIII	Glioma	Peking University	avidin-CD8-CD28-TNFRSF9-CD3zeta	CD3+CD28+IL-2	Lentivirus	BALB/c nude mice	2015
EGFRvIII	Glioma	Zhengzhou University People's Hospital	scFv-hinge-TM-ICOS-CD3ζ	CD3+CD28+IL-2	Lentivirus	BALB/c nude mice	2013
EpCAM	Prostate cancer	Peking Union Medical College and Chinese Academy of Medical Sciences	scfv-CD28-CD3zeta	CD3+CD28+IL-2	Retrovi-ruse	NOD/SCID mice	2015
ErbB2	Breast/ovarian cancer	Wenzhou Medical College	scFv-Fc-CD28-CD3z	PHA-L+CD3+IL-2	Electro-poration	Balb/c nude mice	2014
ErbB2	Breast cancer	Wenzhou Medical College	scFv-Fc-CD28-CD3	PHA-L+CD3+IL-2	Electro-poration	*Vitro*	2012
ErbB2	Breast cancer	The Second Military Medical University	scfv-cMyc tag-CD28-CD3zeta	CD3+CD28+IL-2	Retrovirus	BALB/c mice	2009
NKG2D ligands	Gastric cancer peritoneal metastasis	Shandong Cancer Hospital and Institute	NKG2D-CD28-CD3zeta	CD3+CD28	Lentivirus	*Vitro*	2015
GPC3	Hepatocellular carcinoma	Renji Hospital, Shanghai Jiaotong University	eGFP-scfv-CD8a hinge-CD28TM-CD28-CD137-CD3zeta	CD3+CD28+IL-2	Lentivirus	NOD/SCID mice	2014
LMP1	Nasopharyngeal carcinoma	Nanjing Medical University	scfv-IgG1CH2CH3-CD28-CD3zeta	CD3+CD28+IL-2	Lentivirus	BALB/c nude mice	2014
VEGFR-1	Breast/NSCL/cervix cancer	West China Hospital, Sichuan University	scfv-hinge-IgG1 Fc-CD4TM-CD3zeta	CD3+IL-2	Electro-poration	NOD-SCID BALB/c mice	2013

a) The published results are collected from PubMed search results with combinations including "chimeric antigen receptor" and "CAR-T cells", and the results are collected before 15th January 2016. Due to the limited collection approach, the results might not be comprehensive, please understand if there are any omissions.

difficulty of gene modification, and the safety and efficacy of these newly developed constructs have yet to be established in human studies (Chmielewski and Abken, 2015). In China, the next-generation CARs are also being developed at present, and hopefully, they will help improve the safety and efficacy of CARs, thus taking Chinese CARs to the next level.

CAR-T CELL CULTURE AND ENGINEERING

In China, T cells used for CAR modification are acquired from peripheral blood mononuclear cell (PBMC) culture. To activate T cells, combination such as OKT3+CD28, OKT3+ interferon gamma (IFN-γ) or phytohemagglutinin (PHA)-L+IL-2 are used for days of stimulation, after which, the cells are maintained with IL-2 (Table 1). The whole culture process lasts about 10–14 d (Gao et al., 2014; Wang et al., 2014, 2015). The stimulation process aims to make T cells susceptible to viral transduction, especially retrovirus, and to achieve a large number of T cells. T cells infused back to patients are expanded about 100–1,000 fold *in vitro*, which tend to induce T cell terminal differentiation. Preclinical studies abroad showed that the adoptive transfer of T cells with the less differentiated naïve and central memory phenotype have greater persistence and antitumor effects than the transfer of terminally differentiated effector cells (Gattinoni et al., 2005, 2011; Hinrichs et al., 2011). To achieve the less differentiated cells, one approach is to iso-

late the T_N (Naïve T) or T_{CM} (central memory T) cells (Wang et al., 2012), while the other approach is to optimize the culture condition to accommodate the less differentiated cells. Recently, γ-chain cytokines including IL-7, IL-15, and IL-21 have been shown to better preserve the naive and central memory phenotype of T cells compared to IL-2 (Perna et al., 2014; Singh et al., 2011; Xu et al., 2014). The usage of these cytokines could be in favor of the T cell culture in China. Additionally, because the less differentiated T cells have greater proliferative potential and tumoricidal capacity *in vivo*, the culture time and transfer dose of T cells might be reduced. This will simplify the practicalities of CAR-T cell production and hopefully improve the whole delivery system.

The gene modification methods of CAR-T cells in China are mainly lentivirus (Wang et al., 2015), retrovirus transduction (Deng et al., 2015) and plasmid electro-transfection (Wang et al., 2013) (Table 1). Virus transduction is favorable for inserting the gene of interest into the target cell genome and precipitating continuous expression. While plasmid transfection enables the transient expression of genes, stable expression can be achieved through additive antibiotic screening. The virus system is more advantageous for creating stable CAR expressing T cell and prolonging CAR expressing T cells *in vivo*. Although there is a remote possibility that retrovirus or lentivirus conducted gene insertion could induce oncogenic-associated mutations, evidences from clinical studies till now indicate that this approach is generally safe (Barrett et al., 2014; Gill and June, 2015). Moreover, transposon systems such as sleeping beauty (Deniger et al., 2015; Singh et al., 2015) and piggybac (Nakazawa et al., 2011; Ramanayake et al., 2015) have been explored for CAR modification and used in hematological malignancy therapies abroad, the clinical safety and efficacy of which are under evaluation. The temporary expression of CAR conducted by recently developed mRNA transfection is also accepted because it will not cause insertional mutagenesis. Also, the limited expression time is preferable for CARs targeting antigens with potential risk (Kenderian et al., 2015; Krug et al., 2014). Theoretically, lentiviruses and transposons can transfect undivided cells, and thus avoid stimulation induced T cell differentiation; however, stimulation with mitogenic agents is still needed to change the resistant state of primary T cells (Cheadle et al., 2014; Morgan and Kakarla, 2014). Besides, transcription activator-like effector nuclease (TALEN) system is used to knock out TCR and CD52, in order to achieve "off the shelf" CAR-T cells (Couzin-Frankel, 2015; Poirot et al., 2015). Other gene editing tools such as CRISPR/Cas9 are also being used to develop more favorable CAR-T phenotype. An approach that is able to efficiently transfect T cells without causing T cell differentiation is ideal. The present gene delivery systems may be satisfactory for mouse studies and clinical trials, but they are still expensive and difficult for steady large-scale production. In general, an optimal gene delivery system is crucial for the CAR industry and clinical efficacy, the improvement of which will promote the development of the industry as a whole. Considering that CAR-T therapy is a personalized treatment strategy and that the engineering and transportation processes are crucial, it is necessary to build multiple regional clinical trial stations. Cooperation between multiple local research institutions and hospitals would help in the building of this CAR-T clinical trial network.

CAR-T CELLS TARGETING HEMATOLOGICAL MALIGNANCIES

CAR-T cells are used in treating hematological malignancies including acute lymphocytic leukemia, chronic lymphocytic leukemia, lymphoma and multiple myeloma. The target antigens include CD19 (Dai et al., 2015), CD20 (Wang et al., 2014), CD30, CD33 (Wang et al., 2015) and CD138 (Guo et al., 2015) (Table 2). CD19 is the most popular target in China, where clinical trials are being conducted in institutions including the Chinese PLA General Hospital, Affiliated Hospital to Academy of Military Medical Sciences and The Second Hospital of Anhui Medical University etc.. According to PubMed search results, the published clinical data in China are mainly from the Chinese PLA General Hospital. To date, three clinical studies have been reported. In a pilot clinical trial, autologous or donor-derived T cells modified to express CAR19 were used to treat patients with relapsed or chemotherapy-refractory B-cell lineage acute lymphocytic leukemia (B-ALL). Nine patients were enrolled, six of whom had extramedullary involvement. Five patients had objective hematopoietic system and extramedullary tissue response for 2–9 months. Cytokine release syndrome (CRS) was observed in most cases. In two patients who received donor-derived cells, grade 2–3 graft-versus-host disease (GVHD) was observed (Dai et al., 2015). Moreover, patients with chemotherapy refractory advanced diffuse large B cell lymphomas (DLBCL) were treated with CAR20 T cells. Two patients with no bulky tumors were enrolled, and one achieved a durable complete remission for 14 months while the other achieved a 6-month regression. Five patients with bulky tumor burdens were also treated, and three of them achieved tumor regressions lasting 3–6 months. The associated toxicities mainly included cytokine release syndrome, tumor lysis syndrome, alimentary tract hemorrhage and intrapulmonary inflammation surrounding extranodal lesions (Wang et al., 2014). Also, one patient with acute myeloid leukemia (AML) was treated with CAR33 T cells. A marked decrease of blasts in the bone marrow was observed two weeks after therapy, and there was a gradual increase until disease progression occurred nine weeks after therapy. Cytokine release syndrome was also observed in the patient (Wang et al., 2015). In these studies, it was observed that CAR-T cells reached their peak 2–3 weeks after cell infusion, and this

Table 2 Clinical trials in China infusing CAR-T cells[a)]

Antigen	Tumor target	Sponsor	Phase	Number enrolled	NCT Number	Study start
CD133	Liver cancer; Pancreatic cancer; Brain tumor; Breast cancer; Ovarian tumor; Colorectal cancer; Acute myeloid and lymphoid leukemias	Chinese PLA General Hospital	Phase 1	20	NCT02541370	2015
CD138	Relapsed and/or chemotherapy resistant multiple myeloma	Chinese PLA General Hospital	Phase 1 / Phase 2	10	NCT01886976	2013
CD19	Acute lymphoblastic leukemia	Affiliated Hospital to Academy of Military Medical Sciences; Peking University	Phase 1	5	NCT02186860	2015
CD19	Non-hodgkin lymphoma; Mantle cell lymphoma	Chinese PLA General Hospital	Phase 1 / Phase 2	2	NCT02081937	2014
CD19	Relapsed and/or chemotherapy refractory B-cell malignancy	Chinese PLA General Hospital	Phase 1	12	NCT01864889	2013
CD19	B-cell lymphomas	Peking University; University of Florida	Phase 1 / Phase 2	20	NCT02247609	2014
CD19	Relapsed/refractory leukemia and lymphoma	Shanghai Tongji Hospital, Tongji University School of Medicine	Phase 1 / Phase 2	40	NCT02537977	2015
CD19	Chronic lymphocytic leukemia; Acute lymphocytic leukemia; Lymphoma	Shenzhen Second People's Hospital; Shenzhen institute for innovation and translational medicine	Phase 1	36	NCT02456350	2015
CD19	Leukemia; Lymphoma	Southwest Hospital	Phase 1	45	NCT02349698	2014
CD19	Recurrent or refractory B-cell tumor	Second Military Medical University	Phase 1 / Phase 2	20	NCT02644655	2015
CD19	Leukemia; Lymphoma	Beijing Doing Biomedical Co., Ltd.; First Hospital of Jilin University	Phase 1	100	NCT02546739	2016
CD19	Recurrent or stage III/IV diffuse large cell lymphoma; Follicular lymphoma; Mantle cell lymphoma	Xinqiao Hospital of Chongqing	Phase 1 / Phase 2	20	NCT02652910	2015
CD30	Hodgkin's lymphoma; Non-Hodgkin's lymphoma	Chinese PLA General Hospital	Phase 1 / Phase 2	30	NCT02259556	2014
CD30	Lymphomas	Peking University; University of Florida	Phase 1 / Phase 2	20	NCT02274584	2014
CD33	Relapsed and/or chemotherapy refractory adult myeloid leukemia	Chinese PLA General Hospital	Phase 1 / Phase 2	10	NCT01864902	2013
CEA	Lung cancer; Colorectal cancer; Gastric cancer; Breast cancer; Pancreatic cancer	Southwest Hospital	Phase 1	75	NCT02349724	2014
EGFR	Advanced EGFR-positive solid tumors	Chinese PLA General Hospital	Phase 1 / Phase 2	10	NCT01869166	2013
EGFR	Advanced glioma	RenJi Hospital	Phase 1	10	NCT02331693	2014
EphA2	EphA2 positive malignant glioma	Fuda Cancer Hospital	Phase 1 / Phase 2	60	NCT02575261	2015
GPC3	Hepatocellular carcinoma	RenJi Hospital	Phase 1	10	NCT02395250	2015
HER-2	Chemotherapy and/or HER-2 antibody inhibitor therapy refractory advanced HER-2 positive solid tumors	Chinese PLA General Hospital	Phase 1 / Phase 2	10	NCT01935843	2013
HER-2	Breast cancer	Fuda Cancer Hospital	Phase 1 / Phase 2	60	NCT02547961	2015
Mesothelin	Malignant mesothelioma; Pancreatic cancer; Ovarian tumor; Triple negative breast cancer; Endometrial cancer; Other mesothelin positive tumors	Chinese PLA General Hospital	Phase 1	20	NCT02580747	2015
MUC1	Malignant glioma of brain; Colorectal carcinoma; Gastric carcinoma	PersonGen Biomedicine (Suzhou) Co., Ltd.; Anhui General Hospital of Armed Police Forces	Phase 1 / Phase 2	20	NCT02617134	2015

a) The clinical trials are collected from clinicaltrial.gov., search results with combinations including "chimeric antigen receptor" and "CAR-T cells", and the results are collected before 15th January 2016. Due to the limited collection approach, the results might not be comprehensive, please understand if there are any ommissions.

was maintained for a couple of months. Broadly speaking, these clinical results are highly inspiring with multiple reports of objective clinical responses in patients with advanced, chemo-refractory hematological malignancies treated with CAR modified T cells.

Some clinical issues are also addressed in these studies. Preconditioning therapy has been shown to enhance the efficacy of CAR-T therapies. On one hand, the lymphodepletion treatments could induce the clearance of Tregs and help the expansion of infused T cells in a lymphocyte homeostatic fashion (Rosenberg and Restifo, 2015). On the other hand, the de-bulking treatments could reduce the tumor burden and disrupt the tumor structure, thus enhancing the trafficking of T cells and mitigating tumor lysis syndromes (Lee et al., 2014). Toxicities including cytokine release syndrome, tumor lysis syndrome, neural toxicity and

inflammation accompany with clinical efficiencies. To ensure the safety of CAR-T cell transfusion, the cell doses are used in an escalating manner. Etanercept (anti-TNFa), tocilizumab (anti-IL-6R) or corticosteroids are used for controlling cytokine release syndromes (Wang et al., 2014). Organ distribution is decisive for CAR-T cells to interact with tumor cells. It is reported that CAR copies can be detected in the bone marrow, cerebrospinal fluid and nodules, but cannot be detected in adipose and extramedullary tissues. The distribution patterns of CAR-T cells provide an explanation for the different clinical responses after cell infusions. Furthermore, donor-derived T cells can cause GVHD (graft-versus-host disease) for some patients, indicating that CAR-T cells should be used with caution for patients who have received allogeneic hematopoietic stem cell transplantation (allo-HSCT) (Dai et al., 2015). For these patients, EBV specific T cells (Pule et al., 2008; Terakura et al., 2012) or TCRαβ-knocked out CAR-T cells (Poirot et al., 2015) are possible solutions. EBV specific T cells or TCRαβ-deficient T cells are not able to recognize host antigens, and thus, the attack of host tissues can be avoided (Cruz et al., 2013; Poirot et al., 2015; Valton et al., 2015).

Generally speaking, hematological malignancies are most responsive to CAR-T therapy so far. Inspiring results of CAR-T-19 therapy from the Memorial Sloan Kettering Cancer Center (Davila et al., 2014), the National Cancer Institute (Lee et al., 2015) and the University of Pennsylvania (Maude et al., 2014) have been reported. In accordance with studies in China, they have all reported the feasibility and anti-tumor efficacy of CAR-T cells against hematological tumors. According to the data reported, complete remission (CR) rates in China are not as high as in the US (Maude et al., 2015). The patients' background largely accounts for this difference. In China, the participants recruited in clinical trials are often more heavily treated and in their later stages as compared to the participants in the US. Clinicians in China would largely improve this situation if they would encourage patients relapsed or refractory from standard treatments to participate in reliable clinical trials.

There are some attributes of hematological malignancies that make them suitable targets for CAR-T therapy. In hematological malignancies, the tumor cells are in the circulation system, which makes them easier for T cells to intercept and more accessible for evaluation (Porter et al., 2015). Tumor-specific antigen expression is relatively limited. For example, CD19 is restrictedly expressed by B cells, which is favorable for avoiding on target-off tumor toxicity. Since the target cells are in the circulation system, the infused T cells can be directly activated by the antigens and maintained in the system for immune surveillance. Hematological tumor cells always express co-stimulatory molecules, whereas solid tumor cells usually lack such molecules (Maude et al., 2015; Sadelain, 2015; Turtle, 2014). With these advantageous features for CAR-T therapy, the clinical outcomes of hematological malignancies are quite encouraging. In contrast, due to their lack of natural favorable characteristics, non-hematological malignancies remain as challenging targets for CAR-T therapy.

CAR-T CELLS TARGETING NON-HEMATOLOGICAL MALIGNANCIES AND COMBINATORIAL STRATEGIES

In contrast to the success observed in the treatment of hematological malignancies, there is a lack of encouraging clinical data about CAR-T therapy for solid tumors. In China, according to published results, T cells are modified with CARs targeting EpCAM (epithelial cell adhesion molecule) for prostate cancer (Dai et al, 2015), EGFRvIII (epidermal growth factor receptor variant III) for glioma (Liu et al., 2015; Shen et al., 2013), LMP1 for nasopharyngeal cancer (Tang et al., 2014), GPC-3 (glypican-3) for hepatocellular carcinoma (Gao et al., 2014), and VEGFR-1 (vascular endothelial growth factor receptor 1) (Gao et al., 2015) and EGFR (Zhou et al., 2013) for multiple tumors (Table 1). These preclinical studies have shown potential and have paved the way for the evaluation of CARs in clinical settings. According to the clinical trial registrations, CARs targeting EGFR, Her-2 (human epidermal growth factor receptor-2), EGFRvIII and GPC-3 for multiple solid tumors are under investigation (Table 2). To improve the efficacy of CARs in targeting non-hematological malignancies, there are some issues that need to be addressed.

Tumor antigens used as CAR targets mainly include mutated antigens, lineage antigens and over-expressed antigens. In hematological malignancies, especially B cell malignancies, the most successful target antigen CD19 is lineage restricted. The targeting of such a lineage limited antigen is relatively safe, and its eradication can lead to consequences that are remediable (Maude et al., 2015; Sadelain, 2015). For solid tumors, mutated antigens are attractive targets. EGFRvIII is the most common variant of EGFR observed in human tumors, and particularly in glioblastoma. CARs targeting EGFRvIII have been developed for glioblastoma, after the demonstration of safety and efficacy in mouse models, phase I clinical trial is underway (Johnson et al., 2015; Morgan et al., 2012). Mutated antigens and lineage antigens are favorable but rare, and most of the available antigens are over-expressed antigens such as EGFR, Her-2 and GPC-3. These antigens expressed in tumor cells are also expressed in normal cells at lower level. This makes the on target-off tumor toxicity a critical issue (Hinrichs and Restifo, 2013; Kakarla and Gottschalk, 2014; Song et al., 2015). ScFv affinity optimization is helpful for solving this problem (Caruso et al., 2015; Liu et al., 2015). The future development of CARs targeting solid tumor antigens in China may also benefit from scFv affinity optimization. Moreover, the safety switch iCaspase 9 (Budde et al., 2013) and iCAR

strategy (Fedorov et al., 2013) also have potential to divert off-target immune responses.

Effective CAR-T therapy requires not only the generation of cancer-specific T cells, but also that these T cells can physically contact cancer cells directly. Solid tumors are known to be comprised of abundant extracellular matrix and tumor-associated stroma cells. The structure and microenvironment of solid tumors makes them privileged for T cell exclusion. Such a circumstance makes T cell traffic a herculean hurdle for CAR-T therapy in solid tumors (Joyce and Fearon, 2015). To improve the trafficking of adoptive transferred T cells, some approaches have been developed abroad in recent years. Prasad et al. compared conventional systemic intravenous and regional intra-pleural administration for the treatment of malignant pleural mesothelioma. They found that intra-pleural T cell administration was superior against pleural disease and even disseminated tumor sites (Adusumilli et al., 2014). For solid tumors residing in elastic cavities, this regional administration approach may be an advantageous option. T cell traffic in the tumor blood vessel requires the interaction between chemokines and chemokine receptors (CCR) (Molon et al., 2011). Edmund et al. found that CAR-T cells with chemokine receptor expression had superior tumor localization and antitumor efficacy as compared with conventional CAR-T cells (Moon et al., 2011). Thus, for the treatment of tumors secreting an abundance of certain types of chemokines, the modification of CAR-T cells to express matched CCRs is beneficial. Once T cells enter the tumor mass, extracellular matrix (ECM) in stroma-rich solid tumors forms another defense against T cell attacks. To ensure the continuous contact of T cells and tumor cells, T cells must be able to degrade ECM (Tan et al., 2015). Ignazio et al. engineered CAR-T cells to constitutively express heparanase, an enzyme that is able to degrade heparan sulfate proteoglycans, which are the primary components of ECM. Heparanase significantly promotes tumor infiltration and the antitumor activities of CAR-T cells (Caruana et al., 2015). This study provided a potential approach that may enhance the efficiency of CAR-T cells, especially in stroma-rich solid tumors. The strategies above may help improve CAR-T cell structure in China, but they also need to be used with caution to prevent potential off-tumor toxicities.

When CAR-T cells enter the tumor mass, they confront with multiple immune suppressive factors, including hypoxia, low pH, the lack of metabolic components, tumor-derived cytokines or metabolites, immune suppressor cells, and inhibitory receptors (Beavis et al., 2015; Gajewski et al., 2013). Though co-stimulatory molecules such as CD28 and CD137 can maintain the survival of CAR-T cells, they do not sufficiently protect CAR-T cells from the inhibition induced by the tumor microenvironment. It is reported that CAR-T cell hypofunction was reversible when the cells were isolated from the tumor. Multiple factors contribute to the hypofunction of T cells, including intrinsic T cell inhibitory enzymes and the expression of surface inhibitory receptors (PD-1, LAG3, TIM3, and 2B4) (Beavis et al., 2015; Enblad et al., 2015; Moon et al., 2014). The interception of immune suppressive factors such as indoleamine 2,3-dioxygenase (IDO) and PD-1 is proved to enhance the antitumor activity of CAR-T cells (John et al., 2013; Ninomiya et al., 2015). Inhibition of Akt signaling is also proven to promote the generation of superior tumor-reactive T cells by studies in China and abroad (van der Waart et al., 2014, 2015; Wu et al., 2015). Except for these combinatorial therapies aimed at counteracting immune suppressive effects, other promising combinatorial strategies have also been evaluated. Epigenetic modifiers, capable of up-regulating tumor antigen expression and increasing tumor antigen intensity, are beneficial for the eradication of tumor cells by CAR-T cells (Anurathapan et al., 2014). Whole Cell vaccine is also reported to enhance antitumor responses of CAR-redirected cytotoxic T lymphocytes recently (Caruana et al., 2015). Chemotherapy is already used as pre-conditioning therapy in hematological malignancies. For solid tumors, chemotherapy and other tumor cytotoxic agents are able to change the tumor structure by inducing tumor cell death, affecting tumor stromal cells, and even reducing myeloid-derived suppressor cells (MDSCs) (Mahoney et al., 2015). These changes in the tumor microenvironment induced by tumor cytotoxic agents suggest it as a synergistic companion for CAR-T therapy. Additionally, new combinations of CAR-T therapy and other current approaches including radiotherapies and antibodies targeting tumor cells also require further exploration.

FINAL THOUGHTS

CAR-T therapy has brought a new hope to cancer patients around the world, including China. However, there are still issues to be solved for CAR-T cell application, including side effects such as on target-off tumor effect, CRS and neural toxicity, optimal cell dosage and infusion frequency, enhancement of specificity and efficacy for solid tumors, and recurrence and treatment post CAR-T therapy. Adoptive immune cell therapy is categorized as one of the third kind medical technologies in China. A notice was released by the National Health and Family Planning Commission of PRC (NHFPC) on June 29th, 2015. This notice declares the cancelation of the approval procedure of the third kind medical technologies clinical application in China, and in its place, a registration system has been adopted. Medical organizations are responsible for the clinical application of the third kind medical technologies themselves. This notice made CAR-T therapy more accessible to general medical organizations; however, due to its potential side effects and toxicities, the clinical application of CAR-T cells should be pursued with caution. Since the understanding of CAR-T therapy is limited so far, large-scale general hospitals with both medical and research orientations are favorable to the

clinical application and development of CAR-T therapy. Since the process of CAR-T therapy involves both cell processing approaches and gene modification products, it is presumable that the approval of this therapy would require the certification of both in the future. For the future industrialization of CAR-T therapy, the cooperation of enterprises and medical organizations will be helpful. In the US, Juno cooperates with the University of Pennsylvania Medical Center and Kite collaborates with National Cancer Institute. In China, Cellular Biomedicine Group partners with the Chinese PLA General Hospital. It is estimated that CAR-T therapy would cost tens of thousands of RMB for one patient; therefore, large-scale clinical trials of CAR-T therapy would be in need of a large amount of financial support. Wall Street has already provided financial support for CAR-T therapy in the US (Barrett et al., 2015). Although the use of CAR-T therapy is still in the early phases in China, we believe that the Chinese financial market will also lend a hand for CAR-T therapy in the near future. In recent years, basic researches aim to improve CAR-T efficacy, safety, universality and applicability for treating solid tumors have made achievements in other nations worldwide. Institutes or enterprises abroad have grown to be skilled in their particular areas. In China, though we have the second most CAR-T clinical trials for now, we are still in lack of self-dependent innovations so far. It is presumable that if "CARs" made in China want to go further, basic research aim to understand and improve the present CAR is indispensable. To accelerate CAR-T therapy in China, we need to strengthen the independent research and development of CAR-T cells, establish the safe and standard clinical research systems, and actively carry out the relevant knowledge and application training of clinicians. The structure of CAR at present has achieved the recognition and stimulation of T cells. This has already exhibited surprising anti-tumor effects. We believe that the dis-inhibition of CAR-T cells and other combination strategies have great potential for promoting this therapy forward.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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